Patterns of DNA Methylation across the Leptin Core Promoter in Four Diverse Asian and North American Populations

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ABSTRACT

DNA methylation is the most widely studied of epigenetic mechanisms, with environmental effects recorded through patterned attachments of methyl groups along the DNA that are capable of modifying gene expression without altering the DNA sequencing. The degree to which these patterns of DNA methylation are heritable, the expected range of normality across populations, and the phenotypic relevance of pattern variation remain unclear. Genes regulating metabolic pathways appear to be vulnerable to ongoing nutritional programming over the life course, as dietary nutrients are significant environmental determinants of DNA methylation, supplying both the methyl groups and energy to generate the methylation process.

Here we examine methylation patterns along a region of the metabolic gene leptin (*LEP*). *LEP*'s putative functions include regulation of energy homeostasis, with its signals affecting energy intake and expenditure, adipogenesis and energy storage, lipid and glucose metabolism, bone metabolism, and reproductive endocrine function. A pattern of differential methylation across CpG sites of the *LEP* core promoter has been previously identified; however, any consistency of pattern or its phenotypic significance is not fully elucidated among populations. Using DNA extracted from unfractionated white blood cells of peripheral blood samples, our pilot study, divided into two parts, examined the significance of variation in DNA methylation patterns along the leptin core promoter in four populations (phase 1) and used biomarkers reflecting leptin's functional process in two of those populations, western Buryat of Siberia and the Mennonite of central Kansas, to investigate the relevance of the ethnic variation identified in the DNA methylation (phase 2).

LEP's core promoter region contains both the binding site for C/EBPα (CCAAT/enhancer binding protein alpha), which tempers the final step in adipocyte maturity and capacity to synthesize leptin, and the TATA motif controlling leptin synthesis. Previous studies report that increased methylation in this region is correlated to decreased gene expression, suggesting tissue-specific methylation variation at this region (Melzner et al. 2002). We hypothesized that evidence of nutritional epigenetic programming would be identified through variation in patterns of DNA methylation and that functional relevance of that variation among populations would be identified through biomarkers that reflect leptin's metabolic signals: serum leptin levels, lipoproteins of the lipid transport system, and anthropometric measures.

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In phase 1, our combined analyses of 313 individuals documented a distinct and consistent overall pattern of differential DNA methylation across seven CpG sites of *LEP* core promoter in all ethnicities and both sexes. This pattern replicates those identified in previous studies, suggesting a conserved core promoter region across populations. Phase 2 analyses of two of the four populations (n = 239), correlating methylation at the C/EBPα transcription binding site (TBS) with metabolic and anthropometric biomarkers reflecting *LEP* roles, showed that stature, which reflects bone growth and remodeling, was significantly and inversely correlated with the percentage of DNA methylation at this site in both sexes. We suggest that variation in DNA methylation along the *LEP* core promoter plays a substantial role in energy signals affecting both adipogenesis and bone metabolism.

Dietary intake and its nutrient content are dynamic environmental factors influencing human gene expression, physiological variation, growth and development, and ultimately adaptation and evolution. Nutritional phenotypes, in part represented by the sum accumulation of gene-by-environment interactions, reflect both parental and the individual's own responses to the environment. These phenotypes are determined through biomarkers of metabolism, adiposity, hormone signals, and genotypes (Mosher 2012; Zeisel et al. 2005). Epigenetics is now believed to archive environmental alternations of gene expression; however, little is known about the effects of variation in epigenetic mechanisms on metabolic biomarkers in normal individuals among diverse populations.

Current epigenetic research emphasizes the mechanisms of DNA methylation, histone modification, and noncoding RNA as an intricate system of ongoing intra- and intercellular signaling and feedback loops required to maintain a stable pattern of cell differentiation and human growth and development during increasingly complex environmental challenges (Jablonka and Lamm 2012; Ptashne 2013). DNA methylation is the most widely studied of these mechanisms. It is interpreted as a method by which effects from environmental factors are registered along the DNA and appear to modify gene expression without altering the DNA sequences. This implies a mediating level of adaptation on gene expression that influences developmental plasticity and phenotypic variation by more rapid, flexible, and even reversible means than the effects of natural selection on DNA sequences (Duncan et al. 2014; Donohue 2013; Gluckman et al. 2007). The process of methylation begins in utero, and its regulation relies heavily on the availability of folate, the one-carbon metabolic pathway, and energy supply (Crider et al. 2012; Stover 2011; Hamid et al. 2009). The stability of epigenetic marks across generations in humans is unclear but appears to be related to their specific role in managing the integrity of the genome and epigenome (Godfrey et al. 2015). Although vulnerable windows during fetal development and early neonatal life are recognized as the primary opportunities for environmental effects on DNA methylation variation (Baie 2015; Bartolomei and Ferguson-Smith 2015; Weaver et al. 2009), patterns of methylation continue under dietary influence throughout a lifetime (Waterland and Jirtle 2004) and are therefore potentially reversible (Vickers 2014; Sebert et al. 2011; Langley-Evans 2009).

Increasing literature documents the importance of metabolic changes driven by novel human dietary choices (Langley-Evans 2009). Studies now indicate variation in gene regulation between humans and nonhuman primates (Blekhman et al. 2014) and identify positive selection in humans for distinct gene promoter regions in glucose metabolism (Haygood et al. 2007) due to the high energy demands of the increased brain function. Hancock et al. (2010) examined genetic variation of adaptive phenotypes in a variety of historically distinct dietary environments and identified folate biosynthesis pathways as one distinct area exhibiting allelic variation across populations. The influence on epigenetic mechanisms in these instances, however, is not known. We question whether epigenetic mechanisms also archive adaptive responses to ancestral diets in a recognizable and heritable pattern in humans. The candidate gene we selected to examine is leptin, which produces a protein signal of the cytokine family (Frühbeck 2006) and is a primary hormonal signal in a complex neural circuitry mediating energy homeostasis (Friedman and Halaas 1998). Stöger

(2006) identified the leptin core promoter as differentially methylated and hypothesized that this variation might also be tissue specific. Heijmans et al. (2008) studied of the Dutch famine of 1944–45 and demonstrated in humans that methylation of the leptin gene is subject to nutritional programming during fetal development and carries sex-specific long-term effects on offspring health. Yajnik (2014) found nutritional programming in genes modulating metabolic responses during the life course, including leptin.

In this study we examined the relevance of variation in DNA methylation across core promoter of the leptin gene (*LEP*) in diverse populations, each with a shared ancestral and environmental history yet from dissimilar ethnicities, climates, lifestyles, and nutritional intake, all of which influence energy balance. We originally hypothesized that significant population differences in patterns of DNA methylation will be evident along the seven CpG sites of the *LEP* core promoter, which contains the C/EBPα transcription binding site (TBS) associated with a final step in adipogenesis for energy storage, and two sites proximal to the TATA motif that impact expression of the leptin signal (Mason et al. 1998). C/EBPα (CCAAT/enhancer binding protein alpha) is a transcription factor that plays many regulatory roles moderating energy homeostasis (Leutz et al. 2011).

We report variation in DNA methylation of the *LEP* core promoter in four populations (n = 88): maternal/offspring dyads from three immigrant populations of Mexican, Ethiopian, and Vietnamese (Mosher and Schanfield 2013), and we add a nonimmigrant reference group of Colorado residents reporting northern European ancestry (Mosher and Schanfield 2015b). The immigrant populations included the mothers who originated in their country of origin and the offspring, reportedly born in the United States. While exact ages were not specified, most of the children were infants and toddlers at the time of DNA testing. Analyses of this maternal/offspring data suggest the existence of both sex-specific variation and generational differences in the DNA methylation of the leptin core promoter (Mosher et al. 2013; Mosher and Schanfield 2015b). These immigrant populations experience rapid and dramatic changes that occur during modern migration. Such precipitous modifications create dietary change derived from abrupt changes in food chain and simulate dietary insults from feast/famine fluctuations and even seasonality in food availability. However, despite the statistically significant variation in degree of DNA methylation at several sites (Mosher et al. 2013; Mosher and Schanfield, 2013), the overall pattern across the seven sites in all the populations remains remarkably stable (Mosher and Schanfield 2015a). We then added paternal data to accompany the maternal/offspring dyads in the Vietnamese and US reference groups. The investigation of those parental/offspring trios of Vietnamese immigrants and a reference group of nonimmigrant parental/offspring of northern European ancestry shows similar outcome in the overall pattern of DNA methylation (Mosher and Schanfield 2015b) despite their dissimilar ethnic and environmental backgrounds (see Figure 1). Here we have expanded this study to include two stable populations with wider age range of participants, whose ancestors remained in their environment over several generations. We hypothesized that the distinct patterns of methylation across the promoter region will reflect adaptation to energy supplies, which may be interpreted through biomarkers reflecting leptin's physiological roles. The first phase of this study compares DNA methylation of the leptin core promoter from the parental/offspring trio data of the Vietnamese and Colorado populations with data obtained from population studies of the Siberian western Buryat and the Kansas Mennonite. The

FIGURE 1. Global locations of the four populations of this study. Two, the Kansas Mennonite of Central Kansas and the reference group—Colorado residents of northern European ancestry—are located by one circle within the United States. The Southeast Asians are reportedly Vietnamese, and the Western Buryat of Siberia are located approximately 65 km north of Lake Baikal.

Table 1. DNA Methylation (mean ± SD) of Seven CpG sites of Southeast Asian Immigrants (*n* = 44) and US Reference Group of Northern European Ancestry (*n* = 45)

CpG Site	Males		Females	
	Southeast Asian	Northern European	Southeast Asian	Northern European
Site 1	14.02 ± 2.03	12.65 ± 1.44	12.24 ± 1.39	12.11 ± 1.12
Site 2	11.32 ± 1.71	11.56 ± 0.96	11.31 ± 1.54	9.77 ± 1.19
Site 3	15.35 ± 1.42	15.80 ± 1.30	14.07 ± 2.01	13.80 ± 1.15
Site 4	**52.39 ± 2.51***	**57.84 ± 2.06***	**50.87 ± 4.17**	**54.44 ± 2.67**
Site 5	29.06 ± 1.92	24.48 ± 1.87	26.02 ± 3.07	23.95 ± 1.60
Site 6	28.57 ± 1.96	29.00 ± 1.93	26.36 ± 2.90	34.95 ± 2.09
Site 7	33.39 ± 11.06	39.58 ± 1.93	34.95 ± 2.92	34.37 ± 1.99
Total methylation	184.11 ± 10.22	190.92 ± 9.28	175.81 ± 17.00	173.83 ± 9.82

Using the Mann-Whitney *U*-test, no site was significantly different between ethnicities. Site 4 (C/EBPα TBS, (shown in boldface) showed >5% ethnic differences and also showed significant differences in sex by generation ($p = 0.002$), with differences between mothers and daughters similar to the pattern of total DNA methylation differences in Figure 3 (Mosher and Schanfield 2015b).

second phase examines biomarkers of plasma leptin, lipids, and anthropometrics with DNA methylation patterns to identify phenotypic relevance in the Buryat and the Mennonite.

Methods

Study Populations

Pilot Phase 1: Vietnamese and Colorado Reference Group of Northern European Ancestry

DNA extracted from unfractionated leukocytes of peripheral blood from two ethnicities of 15 family trios ($n = 89$) was obtained from the George Washington University Forensic Sciences Laboratory. The samples consist of trios of Vietnamese immigrant families: parents born in their country of origin and offspring, mostly toddlers and younger, believed to be born in the US or immigrated to the US soon after birth. The US reference group consists of fifteen trios of northern European ancestry from Colorado, with offspring mostly of age similar to that the Vietnamese offspring. These data, collected in between 1995 and 2003, came with no biomarkers beyond DNA. Little is known regarding the individuals' phenotypes of the original populations, as the DNA is accessed through the paternity studies completed on immigrant and nonimmigrant families. Under these circumstances, no identifiers are available other than reported ethnicity by the mothers, DNA matches to the fathers, generation, and sex. DNA methylation percentages along the seven CpG sites of both populations are listed in Table 1. The use of these data was cleared by institutional review boards at both George Washington University and Western Washington University.

Pilot Phase 2: Buryat and Mennonite

We examined the DNA methylation profiles of two additional populations, the Siberian western Buryat and the central Kansas Mennonite. These two populations previously participated in extensive studies through the Laboratory of Biological Anthropology at the University of Kansas. The studies examined genes, energetics, and nutrition and resulted in similar phenotypic data of fasting plasma leptin and lipid profiles, apolipoproteins, anthropometrics, and dietary profiles. Therefore, to examine the significance of variation in DNA methylation, we compared DNA methylation profiles and biomarkers of these two diverse populations who have not experienced recent migration. Revised institutional review board approval was obtained from Western Washington University for this study of the leptin core promoter in both populations.

The western Buryat peoples, an admixed group of Mongol and Siberian populations formed in the 13th century, are members of the largest indigenous group in Siberia. No longer transhumant, they now live west of Lake Baikal in the national district of Ust'-Ordynsky, where they practice sheep and cattle herding along with some agriculture. Some foods, including flour, juices, alcohol, and hard

candy, are trucked in from Irkutsk. The temperature range of this region is reported from –27°C in winter to above 30°C in summer (Mosher 2002). We obtained data of 77 participants from this Siberian study, ranging in ages from 15 to 74 years of age. Genetic studies identify Buryat exhibiting a lower level of population differentiation than many of the Siberian indigenous populations, although the Ust-Orda population had a moderate level of heterozygosity, suggesting some gene flow into the area (Novoradovsky et al. 1993). DNA samples in this study came from Gakhani, an isolated village within the Okrug. This population experiences high rates of poverty, and the severe economic hardship suggests an outmigration in that village due to the lack of employment. There is a paucity of medical and health records in this area; however, the primary health risk is reported to be essential hypertension. Their population means for plasma lipid profiles are some of the lowest reported, while accounts of morbidity from cardiovascular disease is approximately 28% (V. Spitsyn, personal communication, 2001, as cited in Mosher 2002).

The central Kansas Mennonite are well-defined Anabaptist group originating in the 1600s of Dutch, Swiss, and northern German populations. Their common migration history brought them through the Ukraine and finally to central Kansas in the United States in 1874, where they now practice an agricultural lifestyle (Crawford 2000). The sample size for this study is 152, with ages ranging from 20 to 94 years. A study of the genetics of biological aging began in 1979 at the University of Kansas. The Mennonite report longevity 8–10 years above that of their Kansas neighbors (Crawford 2000); mortality trends on the 1980 study population are reported in Melton et al. (2006). In 2003–2005, researchers from the University of Kansas revisited the population to complete a more in-depth nutrition study (Kansas Nutrition Project) and to collect blood samples for DNA plasma levels of leptin and lipids, along with anthropometrics. Mean plasma lipid levels of this Mennonite population, previously found to be slightly higher than the average US levels in the 1980s (Crawford 2000), now approximate the mean of the US population (Mosher et al. 2004). Demarchi et al. (2005) suggest the possibility of selection pressures on a polymorphism in the apolipoprotein B gene (*Xbal*). This apolipoprotein is the primary protein found in

Table 2. Descriptors of Male and Female Buryat (*n* = 77) and Mennonite (*n* = 152) Samples (mean ± SD)

Characteristic	Males		Females	
	Buryat	Mennonite	Buryat	Mennonite
Trait				
Age	33.0 ± 13*	49.0 ± 16*	32.0 ± 14*	57.0 ± 16*
Body mass index (cm^2/kg)	23.7 ± 4.5*	26.7 ± 3.5*	24.6 ± 5.9	25.1 ± 4.2
Height (cm)	168.63 ± 5.78*	178.88 ± 6.38*	159.22 ± 18.54*	165.56 ± 7.66*
Weight (kg)	67.0 ± 14*	86.0 ± 13*	61.0 ± 15*	68.0 ± 12*
Leptin (ng/ml)	2.54 ± 3*	6.4 ± 4.3*	7.3 ± 4.8*	16.0 ± 8*
LDL (mg/dl)	87.98 ± 36.57*	111.62 ± 29.86*	78.71 ± 25.25*	111.73 ± 38.01*
TG (mg/dl)	91.0 ± 48	120.0 ± 70	81.0 ± 35*	120.0 ± 71*
HDL (mg/dl)	38.75 ± 11.33	43.94 ± 14.60	38.87 ± 17.34*	57.16 ± 15.39*
DNA methylation				
Site 1	14.91 ± 4.12	12.54 ± 3.34	15.66 ± 3.52*	12.82 ± 3.60*
Site 2	11.94 ± 3.85	10.99 ± 3.37	13.02 ± 3.73	11.24 ± 5.15
Site 3	15.06 ± 4.32	14.67 ± 4.39	16.25 ± 4.25	15.01 ± 4.25
Site 4	**61.62 ± 7.51***	**54.41 ± 6.45***	**61.65 ± 7.78***	**55.42 ± 7.99***
Site 5	28.81 ± 6.54	26.31 ± 5.93	29.68 ± 5.74	27.15 ± 9.73
Site 6	27.15 ± 9.73	27.20 ± 5.93	30.86 ± 5.90	30.86 ± 5.90
Site 7	38.02 ± 7.83*	34.28 ± 6.35*	38.97 ± 7.08*	35.06 ± 8.38*
Total methylation	200.22 ± 37.61*	179.03 ± 32.07*	195.98 ± 34.76*	185.29 ± 44.88*

Data are sex stratified and analyzed for ethnic variation. Site 4 (C/EBPα TBS, shown in boldface) remains the only site with >5% differences in DNA methylation between ethnicities. * $p < 0.005$.

low-density lipoprotein, the lipoprotein associated with high delivery of cholesterol around the system. The frequency of this polymorphism is 0.752 in our Mennonite sample but only 0.182 in the Buryat sample (Demarchi et al. 2005; Mosher 2002). The *Xbal* polymorphism was recently documented as not associated with increased heart risks in some European populations (Li 2014). Table 2 provides the descriptors and statistical comparison between each sex in both populations.

Laboratory Analyses

Phenotypes

Blood samples obtained in the Buryat study (1998) and the Mennonite study (2003–2005) for plasma leptin and lipid levels were fasting (8–12 h) draws, acquired by antecubital venipuncture using EDTA Vacutainers. The leptin levels of both populations were analyzed at the Southwest Foundation of Biomedical Research (now the Texas Biomedical Research Institute). Plasma leptin levels were determined by radioimmunoassay (Buryat) and Luminex assay (Mennonite), both supplied by Linco Research, Inc. Coefficient of variation in the radioimmunoassay was reported as 7.2% (Rainwater et al. 1997). Significant differences between the two populations have been identified, with the Siberian Buryat exhibiting lower levels of plasma leptin and plasma lipids than the Mennonite.

Lipid panels, including total cholesterol (TC), high-density lipoprotein (HDL), and triglycerides (TG) for the Buryat were determined at the University of Kansas Medical Center using the Kodak Ektachem system, with HDL measured after precipitation of B-lipoproteins via dextran and magnesium (Leonard et al. 1994). TG was measured after phospholipids were removed. Both very low-density lipoprotein and LDL were determined by Friedwald's equation: very low-density lipoprotein = TG/5; LDL = TC – HDL – TG/5 (Rifai et al. 2008). The Texas Biomedical Research Institute measured lipids for the Mennonite and determined all by their laboratory protocols (Mahaney et al. 2003). This included enzyme assay by Gilford SBA-300 technology and using Boehringer-Mannheim Diagnostics reagents for TC and Stanbio Laboratory reagents for TG. The procedure for HDL in the Mennonite sample was similar to that used for the Buryat sample. During these studies, both facilities were subject to the National Cholesterol Education Program guidelines for lipid analyses, with an expected coefficient of variation of 3% from the reference controls in TC (Caudill et al. 1998) and 3.2% for TG and 5.6% for HDL (Mahaney et al. 2003). As all original samples of both populations were collected in EDTA tubes, all results are reported as plasma levels.

Dietary data were collected through prompted 24-h dietary recall by a Russian translator with the Buryat and then entered in Food Processor software, updated for data of Russian/Siberian foods acquired from the World Health Organization (Mosher 2002). That information restricted this study to macronutrient energy intake only. The Mennonite study collected dietary intake diaries from three nonconsecutive days. The dietary diaries were then entered into NutriBase software (CyberSoft, Inc., Phoenix, AZ, USA) for nutrient profiles. Both programs use data from the US Department of Agriculture and are subject to Atwater System for energy calculations (Merrill and Watt 1973). Percentage macronutrient intakes were calculated using the modified 4:4:9 (carbohydrate:protein:fat) ratio of kilocalories per gram of macronutrient, with the total kilocalorie intake of each macronutrient divided by the total caloric intake and multiplied by 100.

Epigenotypes

The leptin gene (*LEP*), located on chromosome 7q31.3, is the candidate gene for this pilot study, due to its putative role as a mediator of energy homeostasis. We obtained DNA previously extracted from unfractionated leukocytes of peripheral blood of both populations and stored in freezers at the Laboratory of Biological Anthropology, University of Kansas (DNA extraction protocol documented in Melton et al. 2010). Working with Reinhard Stöger at the University of Nottingham, who had completed previous leptin studies in both mice and humans (Stöger 2006), seven CpG sites of the *LEP* core promoter were selected. These sites include one located in the C/EBPα TBS at site 4 and two proximal to the TATA motif at sites 6 and 7 (Figure 2). Analyses of the methylation patterns of *LEP* were completed at the Functional Genomics Lab at the University of Washington. Using Qiagen protocols, DNA bisulfite treatment converted unmethylated cytosine to uracil. Genomic DNA

(250–300 ng) was bisulfite treated using EpiTect Fast DNA Bisulfite Kit (Qiagen, Valencia, CA, USA). Following cleanup of the converted product, DNA was brought up to a concentration of 10 ng/μl, and 20 ng converted DNA was run in the PyroMark polymerase chain reaction (PCR) per manufacturer's protocol (Qiagen). After visual inspection of the product by agarose gel, 8–10 μl PCR product was analyzed using the PyroMark Q24 (Qiagen). Samples that passed the quality control built into the Qiagen system (see Qiagen Resources, https://www.qiagen.com/us/resources/technologies/pyrosequencing-resource-center/pyrosequencing-applications/dna-methylation-analysis/#Valuable) were used. Nineteen samples were competed as duplicates at all seven sites, and the results were statistically indistinguishable. Duplicate sample means ± SD at site 4 for both sets were 53.19 ± 14.79 and 53.21 ± 14.21, with a *t*-test $p = 0.998$.

PCR amplification produced the one amplicon, using forward PCR primer GTGTATATTGAGGGTTTAGGGTTAGTAG and biotinylated reverse primer GGAGGGAGTTGGAGTTAGAAAT. Pyrosequencing was completed using the PyroMark Q24 system. This pyrosequencing method utilizes an enzyme cascade to release pyrophosphate and converts it to light, producing a pyrogram denoting the percentage of DNA methylation identified in the region of the cytosine (C) of the CpG site adjacent to the thiamine (T) (Qiagen Resources). This approach provides accurate, reproducible, and cost-effective quantification of cytosine methylation levels at a given locus, such as the *LEP* promoter, and variation in DNA methylation of CpG islands lying close together has been identified in differential expression of a gene (Tost and Gut 2007).

Statistical Analyses

The percentage of DNA methylation for each CpG site was determined by the percentage of methylation identified in the region of the cytosine of the CpG site adjacent to the thymine,, as described above. Mean differences in DNA methylation, diet, and phenotypic traits were determined by the Mann-Whitney *U*-test and analysis of variance using SPSS Statistics 23.0 (IBM, Armonk, NY, USA). Plasma TG, leptin levels, and stature were natural log transformed (per outcome of Kolmogorov-Smirnov *Z*-test). For analyses of traits with DNA methylation, we used partial Pearson's correlation coefficients adjusted for batch, age, and body mass index (BMI) similar to the approach of Houde et al. (2015). A correlation of all individuals was completed, adjusting for batch, age, and BMI, followed by a sex-stratified approach to acknowledge sexual dimorphism widely reported in both adiposity, lipid profiles, and plasma leptin (Williams et al. 2016; Fuente-Martín et al. 2013, Guerra et al. 2008; Martin et al. 2002; Rosenbaum et al. 2001; Power and Schulkin 2008; Wang et al. 2011).

FIGURE 2. DNA sequence of *LEP* CpG island with this study's seven CpG sites identified. Site 4 documents the C/EBPα transcription binding site, and sites 6 and 7 are proximal to the TATA motif. Data from UCSC Genome Browser on Human, February 2009 (GRCh37/hg19).

Simultaneous regression analyses were completed on sex-stratified data to substantiate the significance of site 4 DNA methylation variation with stature, using two models: with and without total caloric intake. Adding total caloric data reduced the sample size by 11 males and 20 females; however, their ages, plasma leptin levels, and site 4 DNA methylation remained within the 95% confidence interval of the mean for the main sample. Significance in this study is set at $p < 0.001$. We describe variables with significance <0.05 but not <0.001 as trending.

Results

Phase 1 Results

The first group contains two sets of parental/offspring trios, one an immigrant population from Southeast Asia (Vietnam), and one nonimmigrant

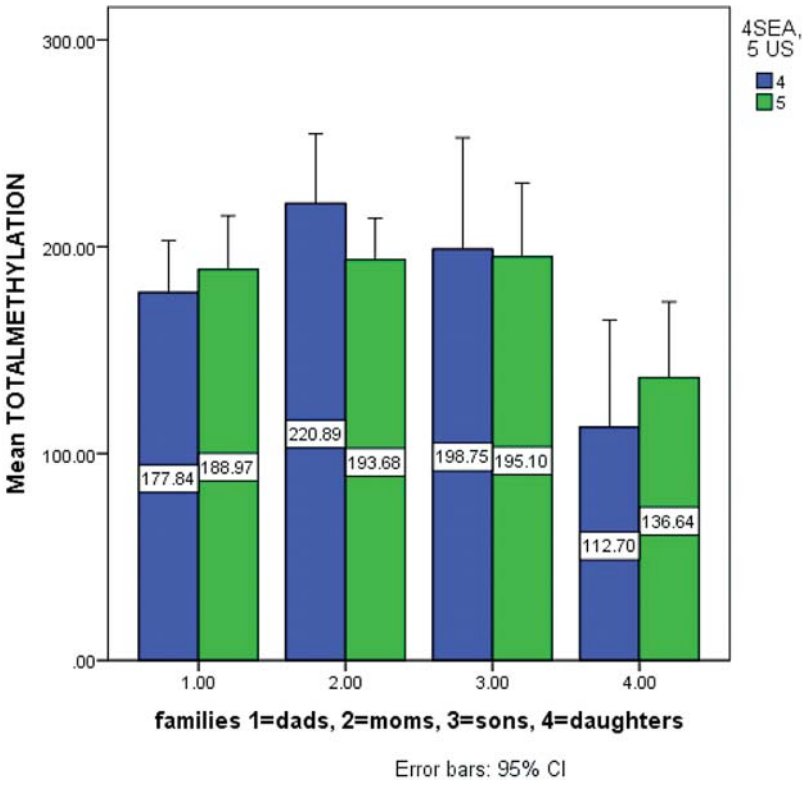

FIGURE 3. Total mean DNA methylation across the seven CpG sites of the *LEP* core promoter in Southeast Asians (Vietnamese) and the US reference group. Only daughters exhibited statistically lower total DNA methylation in both ethnicities.

reference group of Colorado residents of northern European ancestry. The mean percentage of DNA methylation in each of the seven CpG sites and total methylation of the *LEP* core promoter in both ethnicities is shown in Table 1. The trio data carry only descriptors of ethnicity, generation, and sex, and the primary analyses examined these three factors only. Comparing mean DNA methylation by *t*-test, parents exhibited greater mean DNA methylation at sites 2–7 than did the offspring ($p < 0.05$; data not shown). Site 4, the C/EBPα TBS, is most pronounced, with >5% ethnic differences in males, and after sex-stratifying data, no methylation difference between fathers and sons was significant ($r = -0.073$, $p = 0.646$). However, it showed significant differences in sex by generation ($p = 0.002$) between mothers and daughters, with a correlation coefficient of $r = -0.589$ ($p < 0.001$), with daughters showing a lower percentage of methylation in both ethnicities. The differences between mothers and daughters were similar to the pattern of total DNA methylation differences shown in Figure 3 (Mosher and Schanfield 2015b). In Figure 3, total DNA methylation across the seven CpG sites of the leptin core promoter did not differ significantly among fathers, mothers, and sons; however, daughters of both ethnicities exhibited the lowest total methylation ($p < 0.001$). Offspring of both Vietnamese and the northern European reference group had a greater variance in site 4 than did parents, yet differences remained significant only in the females. These findings are reported elsewhere (Mosher and Schanfield 2015b).

Analyzing all four of the populations, variation in DNA methylation at site 4, but not total DNA methylation, was significantly different among Buryat, Mennonite, and Vietnamese males ($p = 0.001$) and Buryat and Vietnamese females ($p = 0.005$).

Phase 2 Results

Because of the lack of biomarkers available with the trio data, the second phase pilot examined only the Buryat and Mennonite samples, which are accompanied by both biological and anthropometric measures (see Table 2). The mean age of Buryat is considerably lower than that of the Mennonite in both males and females. Mennonite males and females were taller and heavier than are Buryat; however, BMI was significantly different in males only. Plasma leptin levels differed, with females having greater levels than males and the Mennonite levels greater than Buryat levels. Plasma lipid levels differed by ethnicity but not by sex in both TG and LDL levels; HDL differed by both sex and ethnicity. HDL was inversely correlated with site 4 degree of DNA methylation after adjusting for ln BMI ($r = -0.287$, $p < 0.001$), with greater levels of HDL correlating with decreased percentage of DNA methylation in females only.

Ethnic differences in degree of DNA methylation >5% are evident only at site 4 in both males and females. Because of the biological significance of site 4, and the significant ethnic variation documented in these pilot populations, we report the phenotype-by-epigenotype results for only site 4. Using the combined data for all participants, and after adjusting for batch, age, and BMI, site 4 was inversely correlated with stature ($r = -0.309$, $p < 0.001$) and trended inversely with plasma leptin levels ($r = -0.146$, $p = 0.031$). After sex stratification of data, and adjusting for batch, age, and BMI, in

males the percentage of DNA methylation at site 4 was inversely correlated with stature ($r = –0.278$, $p = 0.009$) and trended inversely with plasma leptin ($r = – 0.229$, $p = 0.032$); in females, it correlated inversely stature ($r = –0.354$, $p < 0.001$), with plasma leptin ($r = –0.268$, $p = –0.002$), and with HDL ($r = –0.265$, $p = 0.003$). Stature correlated with plasma leptin levels in both males and females ($r = 0.323$ and $r = 0.319$, respectively, with $p < 0.002$). Neither LDL nor TG was significantly correlated with site 4 in males or in females. HDL correlated with stature in females only ($r = 0.259$, $p = 0.003$). Following identical procedures for total methylation of the seven sites, this factor trended negatively with both stature and plasma leptin in males ($r = –0.181$ and $r = –0.195$; both nonsignificant with p-values slightly above 0.05) and correlated negatively with both stature and plasma leptin levels in females ($r = –0.354$ and $–0.268$, respectively; $p < 0.002$). Total DNA methylation was not significantly correlated with lipid profiles in either sex, however.

A simultaneous regression analysis was completed on stature, with factors of age, BMI, plasma leptin residual after adjusting for weight, and methylation of site 4. When all four variables are included in the model, site 4 DNA methylation was significant only in males (standardized $\beta = –0.319$, $t = 3.255$, $p = 0.002$). In females, no factor was significant.

Discussion

This pilot study determined expected normal percentages of DNA methylation across seven CpG sites of the *LEP* core promoter among human populations from different continents and diverse food chains. Additionally, it examined phenotypic relevance of significant variation in DNA methylation at specific CpG sites. The *LEP* core promoter region is identified as differentially methylated (Stöger 2006). It contains both the C/EBPα TBS (at site 4) and the two sites proximal to TATA motif (sites 6 and 7), key promoter elements minimally required for initiating transcription of its protein signal leptin (Kadonaga 2012).

Here we analyze parental/offspring trios of Vietnamese immigrants, a US reference group of nonimmigrants reporting northern European ancestry, and two semi-isolated groups who experience vastly different lifestyles: the Siberia western Buryat, a mixed ancestry of Mongolian tribes and Siberian indigenous groups, and central Kansas Mennonite of Swiss, German, and Dutch ancestry. We replicated an overall pattern of DNA methylation variation previously identified in this region (Figure 4), with C/EBPα TBS consistently showing a higher percentage of methylation than adjacent CpG sites (Marchi et al. 2011). The pattern is similar to that identified by Houde et al. (2015) in a Canadian study of samples of blood and two fatty tissues, subcutaneous and visceral, obtained from 73 patients who underwent surgery for morbid obesity; they reported a similar overall pattern in this region across all the tissues, but a lower percentage of DNA methylation in adipose tissue than in blood These findings support the hypothesis that conservation of methylation patterns across core promoters of primary regulatory regions would be expected among diverse populations (Jiang et al. 2014). Additionally, these findings indicate that the heterogeneity of leukocytes across populations does not result in dramatic variation of methylation findings, because in our study site 4 showed only 5–6% variation, the greatest percent mean variation of all the sites studied here. This epigenetic marker has been shown to be relatively stable across a short time frame in a variety of candidate regions. Leptin resembles the construction of some cytokines, including interleukin-6, shown to have a minimal intra-individual change in methylation after adjusting for percent blood granulocytes, age, and smoking (Byun et al. 2012).

We also found that DNA methylation variation at site 4, the C/EBPα TBS, correlates with height (stature) in both males and females. Here we interpret stature as a surrogate measure representing both developmental growth and bone remodeling. This finding reflects a more extensive function of site 4 methylation beyond its accepted action on maturation of adipocytes for energy storage, as bone growth and remodeling are substantial energy-consuming processes. *LEP*'s putative role is that of regulating energy homeostasis, which balances energy intake, storage, and expenditure through both peripheral and central signaling, managed through a group of leptin receptors, extensive hormonal signaling cascades, and neuronal control (reviewed in Allison and Myers 2014; Ferron et al. 2013; Karsenty and Oury 2012; Scheller et al.

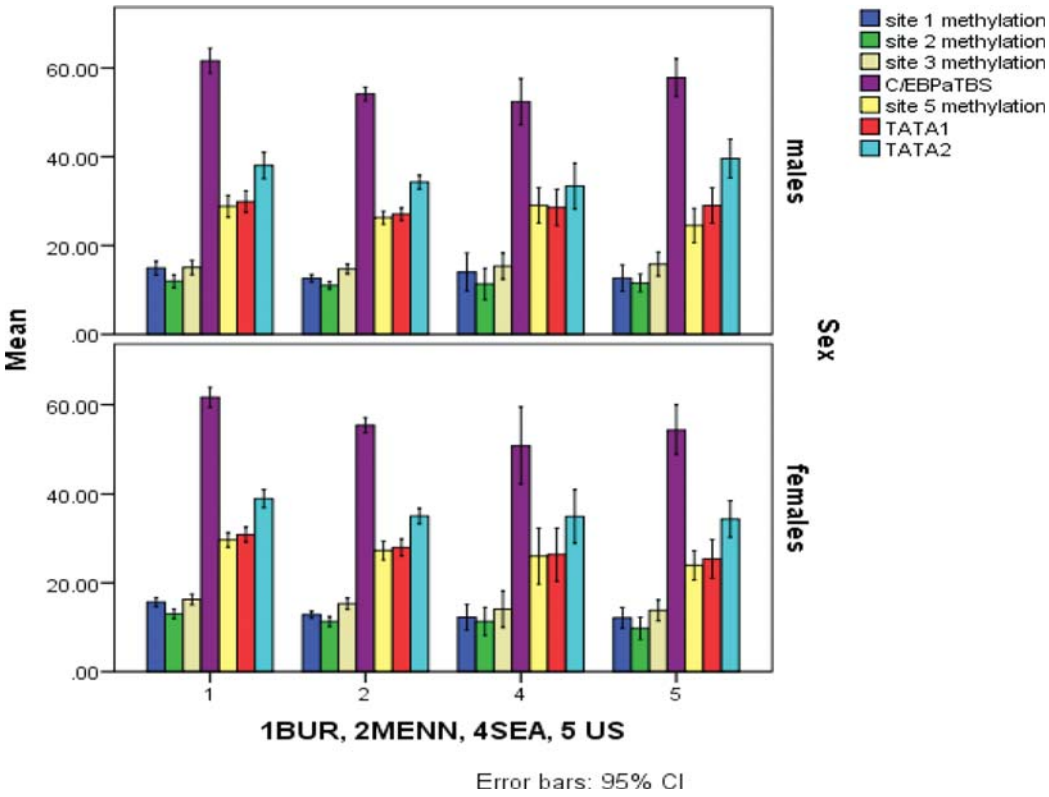

FIGURE 4. Consistent overall pattern of DNA methylation along seven CpG sites in the leptin core promoter in males and females of four populations: 1, Buryat; 2, Mennonite; 4, SE Asia of immigrants; 5, Colorado northern European reference group. Both Buryat and Mennonite are stable populations. This overall pattern replicates studies of this region, with the C/EBPα TBS (site 4) showing the greater degree of DNA methylation across the seven CpG sites.

2010; Mitchell et al. 2009; Frühbeck 2006). Central signaling is accomplish through the hypothalamus and brainstem neural pathways, while peripheral signaling appears affected by nutritional status in human blood. Leptin circulates in two states, free and bound to a soluble receptor. The receptor levels are determined by sex hormones, adiposity, and leptin itself, as well as a circadian influence (Chan et al. 2002). Free leptin reflects body fat mass, while bound leptin appears to have a different regulatory role in energy balance (Brabant et al. 2000). Females exhibit higher free leptin and lower leptin-binding protein than do males (McConway et al. 2000). Here, the sexual dimorphism of fat depots and muscle mass cannot be ignored. Determining free leptin versus bound leptin was not possible in this study, which may explain why the leptin plasma level adjusted for weight was not a predictor of stature in our model. However, our findings can be viewed through an integrative and thrifty physiological model of energy mediation responsive to environmental constraints of energy availability and the individual energy demands related age, sex, and growth and development.

The findings in this pilot study support the hypothesis that the *LEP* core promoter is a region reflecting a conserved pattern of DNA methylation that produces pleiotropic effects (Faggioni et al. 2001). The ultimate fate of multipotent mesenchymal stem cells first located in bone marrow are potentially adipogenic, osteogenic, and even myogenic (Mo et al. 2016), and that these stem cells contain highly functional leptin receptors (Hess et al. 2005). HDL, traditionally inversely linked to adipose tissue and BMI, is also shown to correlate with nonadipose components of the body, such as skeletal mass (Pietrobelli et al. 1999). An animal study showed that HDL promotes proliferation of mesenchymal stems cells derived from bone (Xu et al. 2012). In this study, HDL correlated with stature in females only. C/EBPα is documented in animal studies to control lineage commitment of osteoclasts (Chen et al. 2013). The inverse correlation of DNA methylation variation with stature in both males and females at site 4 suggests that leptin plays a role in the activation of bone remodeling, a highly energy-consuming process. Recent research now supports the concept that bone is also an endocrine organ similar to adipose tissue and plays an active role in energy homeostasis

(Karsenty and Oury 2012), specifically through a reciprocal hormonal relationship of osteocalcin and leptin, regulating insulin and glucose homeostasis (Kanazawa 2015; Schwetz et al. 2012: Ng 2011; Ferron et al. 2010; Kim et al. 2010; Hinoi et al. 2008; Lee and Karsenty 2008). Another study has identified the negative effect of leptin on osteocalcin, which is believed to inhibit insulin and reduce visceral fat (Ferron et al. 2013). The effects of plasma leptin on bone remodeling may in fact be dose dependent and bimodal, producing opposite effects of stimulation and suppression depending upon its levels (Kim et al. 2010; Motyl and Rosen 2012). Effects are also suggested to be sex specific (Dalskov et al. 2015). Much work is needed in this regard, with studies designed to address the intricacies of this relationship in humans from diverse environments and ancestries.

While stature is believed to be an example of a classic polygenic trait with high heritability, a recent genome-wide association study on the genetics underlying this trait found a low percentage (~3–5%) of effect from most of the common variants (Weedon and Frayling 2008), leaving some authors to recommend the study of diverse ethnicities and the redefinition of the phenotype to that of height velocity (Lettre 2011). Recent findings correlate variation in lipid profiles and bone loss (Garg et al. 2014; Go et al. 2014), suggesting that these two functions are mutually constrained in some way; however, studies are inconclusive, with some authors pointing at a paucity of studies examining ethnic variation (Tian and Yu 2015). We have identified here ethnic variation in degree of DNA methylation and in plasma lipid profiles, and that HDL appears related to stature only in females. Further research to determine the role of lipoprotein, specifically that of triglyceride transport, is vital.

The interrelationship of these variables is not fully elucidated in humans, yet results of this study suggest their connection is through a system of energy regulation and adaptive trade-offs. Cell signaling is well beyond the scope of this pilot study. As all data were obtained through past studies not specifically designed to address our question, we are subject to the constraints of models and limitations of past technology. Confounding variables that could not be evaluated in this study include white blood cell heterogeneity, which may affect methylation measures (Adalsteinsson et al. 2012); a limited evaluation of smoking history as well as the effects of maternal smoking (Youselfi et al. 2013); and most important, the role of DNA methylation of leptin receptors. Additionally, obtaining accurate assessments of dietary energy intake using the dietary recall method would benefit from repeated measures, as studies have found an improvement of total energy reporting over several time frames (Yunsheng et al. 2009) or with the use of an active dietary diary, such as used in the Mennonite study, but over a longer time (Willett 1990).

DNA methylation is a complex process, subject to both genetic and environmental influences, and future studies examining the relevance of methylation variation will need to be addressed using specific models that include genetic architecture underlying the relevant cell signaling of the specific metabolic pathways to be examined. Research in human epigenetic mechanisms is subject to many of the limitations of early genetic studies, including small sample sizes, complexities of tissue-specific variation in expression, and discrepancies between conclusions of epigenome-wide studies and findings of individual candidate regions (Heijmans and Mill 2012). The results in this study are subject to the smaller samples, yet they also provide a window to focus further research into the regulation of integrated physiological signals providing an adaptation to energy availability in diverse populations.

Conclusion

This study identifies a conserved differentiated pattern of DNA methylation across seven CpG sites of the *LEP* core promoter among populations varying in age, sex, continental origin, ethnic ancestry, and diet. This pattern replicates those of other studies in both humans and animals, supporting a hypothesis that this DNA methylation pattern is conserved to mediate energy effects across systems. Further research must include an integrated approach of both human physiological systems and the environmental factors that impact individual response and population adaptation. Distinguishing epigenetics through an adaptive model offers the framework through which to explain the epigenotype to phenotype relationship. Energy allocation in developmental physiology, their metabolic effects, and

ultimate phenotypic trade-offs affecting longevity are primary examples of this concept (Symonds et al. 2009). Our understanding of energy regulation is becoming increasingly complex, as is our technological ability to unravel its architecture.

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